Physical and functional association of αLβ2 Integrin (LFA-1) with DNAM-1 adhesion molecule

Kazuko Shibuya[1,2], Lewis L. Lanier[4], Joseph H. Phillips[4], Hans D. Ochs[5], Kenji Shimizu[2], Eiichi Nakayama[3], Hiromitsu Nakauchi[1,6] and Akira Shibuya[1,3,6]

[1]Department of Immunology, Institute of Basic Medical Sciences, and Center for TARA, University of Tsukuba, and CREST (JST), 1-1-1 Tennodai, Tsukuba Science-City, Ibaraki 305 -8575, Japan.

[2]Department of Molecular Genetics, Institute of Cellular and Molecular Biology and [3]Department of Parasitology and Immunology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700, Japan.

[4]Department of Immunobiology, DNAX Research Institute of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, CA 94304

[5]Department of Pediatrics, University of Washington School of Medicine, Seattle, WA 98195-6320.

[6]To whom correspondence should be addressed (e-mail; nakauchi@md.tsukuba.ac.jp, ashibuya@md.tsukuba.ac.jp)

Summary

Whereas ligation of DNAM-1 adhesion molecule triggers cytotoxicity mediated by normal NK and T cells, this function was defective in NK cell clones from leukocyte adhesion deficiency syndrome. However, genetic reconstitution of cell surface expression of LFA-1 restored the ability of DNAM-1 to initiate anti-DNAM-1 mAb induced cytotoxicity, indicating a functional relationship between DNAM-1 and LFA-1. Further studies demonstrated that LFA-1 physically associates with DNAM-1 in NK cells and anti-CD3 mAb stimulated T cells, for which serine phosphorylation of DNAM-1 plays a critical role. In addition, cross-linking of LFA-1 induces tyrosine phosphorylation of DNAM-1, for which the fyn protein tyrosine kinase is responsible. These results indicate that DNAM-1 is involved in the LFA-1-mediated intracellular signals.

Running Title:

Association of LFA-1 and DNAM-1

Introduction

During a successful cellular response, several families of adhesion molecules participate not only in intercellular binding but also in signal transduction for effector cell activation or deactivation (Springer, 1990; Dustin and Springer, 1991; Hynes, 1992; Hogg and Landis, 1993). The β2 integrin adhesion molecules are composed of a common β chain (CD18) that non-covalently associates with α subunits, including leukocyte function-associated antigen-1 (LFA-1; αLβ2, CD11a/CD18), Mac-1 (CD11b/CD18), p150,95 (CD11c/CD18), and αdβ2 (CD11d/CD18) (reviewed inSpringer et al., 1987). The physiological importance of β2 integrin adhesion molecules is revealed by an inherited disorder known as leukocyte adhesion deficiency (LAD) in humans and in cattle, in which defects in the *β2* genes cause a loss of expression of all the β2 integrins, resulting in profound defects in cellular adhesion (Krensky et al., 1985; Anderson and Springer, 1987; Kishimoto et al., 1987; Arnaout, 1990; Shuster et al., 1992). In these patients, many adhesion-dependent functions of leukocytes are abnormal, including adherence to endothelium, aggregation and chemotaxis, phagocytosis, and cytotoxicity mediated by neutrophils, macrophage, NK cells, and T lymphocytes. Mice with disrupted *CD18* genes have demonstrated a phenotype closely resembling LAD patients (Scharffetter-Kochanek et al., 1998).

The αLβ2 integrin, LFA-1 (CD11a/CD18), is expressed on most leukocytes and mediates cell-cell adhesion upon binding to its ligands, the intercellular adhesion molecules (ICAM)-1 (CD54), ICAM-2 (CD102), or ICAM-3 (CD50) (Staunton et al., 1988; Staunton et al., 1989; Fawcett et al., 1992). Circulating peripheral blood leukocytes generally express an inactive form of LFA-1. Once leukocytes are activated, for instance through the T cell antigen receptor (TCR) upon recognition of a peptide antigen or by phorbol 12-myristate 13-acetate (PMA), intracellular signals (referred to as ‘inside-out’ signals) cause a conformational change in LFA-1, resulting in intercellular binding and effector

cell function (van Kooyk et al., 1989; Dustin and Springer, 1989). The cytoplasmic regulatory protein, cytohesin, is a key molecule involved in this process (Kolanus et al., 1996).

Antibody cross-linking of cell surface LFA-1 induces intracellular signals (referred as to 'outside-in' signals) (Kanner et al., 1993; Arroyo et al., 1994), suggesting that ligand binding may also affect cellular functions such as apoptosis, cytotoxicity, proliferation, cytokine production and antigen presentation (Koopman et al., 1994; Moy and Brian, 1992; Springer, 1990; Lanier and Phillips, 1993). Blocking the interaction between LFA-1 and its ICAM ligands induces tolerance against cardiac allografts in mice (Isobe et al., 1992) and studies using mice with disrupted *CD11a* or *CD18* genes have indicated a requirement for LFA-1 in T cell proliferation induced by the TCR/CD3 complex (Schmits et al., 1996; Scharffetter-Kochanek et al., 1998). These observations indicate that LFA-1 not only mediates intercellular binding but also may deliver costimulatory signals in T lymphocytes. In contrast with 'inside-out' signaling, little is known about the intracellular signals initiated by LFA-1 ligation.

The leukocyte adhesion molecule DNAM-1, a member of the immunoglobulin superfamily, is constitutively expressed on the majority of T lymphocytes, natural killer (NK) cells and monocytes (Shibuya et al., 1996). Depending on the experimental conditions, monoclonal antibodies against DNAM-1 can function as agonists, triggering NK or T cell-mediated cytotoxicity and cytokine production, or as antagonists, blocking NK or T cell responses against target cells expressing a putative DNAM-1 ligand (Shibuya et al., 1996). Upon mAb cross-linking, tyrosine residues in the cytoplasmic domain of DNAM-1 are phosphorylated (Shibuya et al., 1996), suggesting that DNAM-1 transduces an activation signal. In this study, we report deficient DNAM-1 function in NK cells from a LAD patient and have explored the relationship between LFA-1 and DNAM-1 signaling.

Results

Defective DNAM-1 function in NK cells obtained from a patient with Leukocyte Adhesion Deficiency (LAD)

mAbs against CD2 and CD16 are able to trigger cytolytic activity when NK cells are co-cultured with target cells expressing Fc receptors (Schmidt et al., 1985, Siliciano et al., 1985). Similarly, anti-DNAM-1 mAb is capable of inducing re-directed cytolysis of Fc receptor-bearing mouse mastocytoma P815 mediated by CTL and NK clones (Shibuya et al., 1996). P815 cells express a murine ICAM that interacts with LFA-1 on human T and NK cells (Cayabyab et al., 1994), suggesting that LFA-1 might be involved in anti-DNAM-1 mAb-induced cytolysis against P815.

To investigate the involvement of LFA-1 in anti-DNAM-1 mAb-induced cytolysis, NK clones were established from the peripheral blood of a patient with leukocyte adhesion deficiency (LAD) (Anderson and Springer, 1987). The NK clones expressed normal levels of CD2, CD16, CD56, and DNAM-1 antigens (not shown), but completely lacked the expressions of the β2-integrins LFA-1 (CD11a/CD18), MAC-1 (CD11b/CD18), and p150/95 (CD11c/CD18) (Fig. 1A). While these NK cell clones demonstrated normal cytolytic activity against certain tumor cell targets, such as K562, we observed that unlike NK clones from healthy donors, they were unable to mediate antibody-redirected cytotoxicity against Fc receptor bearing targets (e.g. P815) in the presence of anti-DNAM-1 mAb (Fig. 1B). This deficiency was selective for DNAM-1, because antibody-redirected killing triggered by mAbs against other activating NK cell surface receptors, for example CD16 (Fig. 1B) or CD2 (not shown) were normal. This finding suggested that DNAM-1-mediated cytolysis by the LAD NK clones may require the participation of LFA-1, either for effector - target cell adhesion or signaling.

Although the loss of β2-integrin expression in LAD disease is attributed to heterogeneous defects in the common β subunit CD18 (Anderson and Springer,

1987), *CD18* gene transfer restores the expression and function of LFA-1 regardless of the site of the molecular defect (Hibbs et al., 1990; Bauer et al., 1998). To examine the requirement of LFA-1 for anti-DNAM-1 mAb-induced cytolysis by NK cells, we infected a LAD NK cell clone with an amphotropic retrovirus containing a wild-type *CD18* cDNA. These retrovirus-transduced NK cells stably expressed LFA-1 (CD11a/CD18) at a level comparable to NK cells from healthy individuals (not shown), but these cells did not express either Mac-1 (CD11b/CD18) or p150/95 (CD11c/CD18) (Fig 1A). Although the precise mechanism is unclear, the dominant expression of LFA-1 was also observed in the previous report after retrovirus-mediated CD18 gene-transfer in LAD cells (Bauer et al., 1998). As demonstrated in Fig 1B, genetic transfer of *CD18* into the LAD NK cell clone completely restored anti-DNAM-1 mAb-induced cytotoxicity against P815 target cells. CD18 was responsible for this function because the anti-DNAM-1 mAb induced killing mediated by the reconstituted LAD NK clone, as well as normal NK cells, was prevented in the presence of $F(ab')_2$ fragments of anti-CD18 mAb.

Our prior observations demonstrated that co-culture of normal NK clones or T clones with anti-DNAM-1 mAb-pre-coated P815 target cells resulted in tyrosine phosphorylation of DNAM-1 (Shibuya et al., 1996). DNAM-1 phosphorylation was completely inhibited in the presence of $F(ab')_2$ fragments of anti-CD18 (not shown). Tyrosine phosphorylation of DNAM-1 was not induced by co-culture of the LAD NK clones with anti-DNAM-1 mAb-pre-coated P815 cells (Fig 1C). However, reconstitution of LFA-1 expression on the LAD NK clone by *CD18* gene transfer restored the DNAM-1 tyrosine phosphorylation induced by co-culture with anti-DNAM-1 pre-coated P815 target cells. Again, this was completely inhibited in the presence of $F(ab')_2$ fragments of anti-CD18 (Fig 1C). These results indicate a functional relationship between LFA-1 and DNAM-1.

LFA-1 physically associates with DNAM-1 in NK cells

The functional relationship between LFA-1 and DNAM-1 led us to examine whether DNAM-1 physically associates with β2 integrins. The CD18-reconstituted LAD NK clones expressed LFA-1 (CD11a/CD18), but not substantial levels of either Mac-1 (CD11b/CD18) or p150/95 (CD11c/CD18). By contrast, normal peripheral blood NK cells express all these β2 integrins (not shown) (Timonen et al., 1988). NK cells isolated from the peripheral blood of a healthy individual were lysed in 1% digitonin buffer and immunoprecipitated with anti-CD18, anti-CD11a, anti-CD11b, or anti-CD11c and the isolated proteins were analyzed by immunoblotting with anti-DNAM-1 mAb. As demonstrated in Fig 2, DNAM-1 was co-immunoprecipitated with CD11a and CD18, but not CD11b or CD11c, suggesting that DNAM-1 associates preferentially with the CD11a α chain or the CD11a/CD18 heterodimer.

CD3 stimulation of T cell induces association of LFA-1 with DNAM-1

Anti-DNAM-1 mAb can trigger cytotoxicity mediated by CTL, as well as NK cells (Shibuya, et al., 1996); however, anti-DNAM-1 mAb does not induce cytotoxicity mediated by resting T cells without prior *in vitro* activation (not shown), raising the issue of whether DNAM-1 and LFA-1 associate in activated, but not resting, T cells. Resting T cells separated from the peripheral blood of healthy donors were lysed in digitonin buffer and immunoprecipitated with anti-CD18 mAb. Strikingly, DNAM-1 did not co-immunoprecipitate with LFA-1 using lysates prepared from resting T cells (Fig 3A). Since antigen binding by TCR or stimulation with anti-CD3 mAb induces a conformational change of LFA-1 (van Kooyk et al., 1989; Dustin and Springer, 1989), we examined whether anti-CD3 mAb induces association of LFA-1 with DNAM-1. As demonstrated in Fig 3A, after T cell stimulation with anti-CD3 mAb DNAM-1 was co-immunoprecipitated with LFA-1. The anti-CD3 induced association of LFA-1 and DNAM-1 was prevented in the presence of a specific inhibitor of PKC (Fig.

3A). In support of the concept that PKC activation is required for LFA-1 association with DNAM-1, we observed that DNAM-1 was co-immunoprecipitated with LFA-1 upon stimulation of resting T cells with PMA in the absence of anti-CD3 mAb (Fig. 3A). Prior studies have shown that activation of PKC causes serine phosphorylation of DNAM-1 on residue 329 in the cytoplasmic domain and that S^{329} phosphorylation of DNAM-1 increases its ligand binding activity (Shibuya et al., 1998). Therefore, resting T cells were metabolically labeled with [^{32}P]orthophosphate, stimulated with anti-CD3 mAb or PMA, lysed, and immunoprecipitated with anti-DNAM-1 mAb. As shown in Fig. 3B, treatment of resting T cells with either anti-CD3 mAb or PMA resulted in phosphorylation of DNAM-1, suggesting that phosphorylation may play an important role in the association of DNAM-1 with LFA-1. By contrast, stimulation with pervanadate did not induce or increase the association of LFA-1 with DNAM-1 in resting or anti-CD3 activated T cells, respectively (Fig 3C), indicating that tyrosine phosphorylation of DNAM-1 or LFA-1 may not be required for this interaction.

S^{329} of DNAM-1 plays a critical role for the association of LFA-1 with DNAM-1 in T cells

Unlike the situation with resting T cells, DNAM-1 was co-immunoprecipitated with LFA-1 in lysates prepared from the human T and NK cell leukemias Jurkat and NKL, respectively (Fig 4A), as well as from freshly isolated NK cells (Fig 2), suggesting a constitutive associate of DNAM-1 and LFA-1 in these cell types. Our prior findings that activated PKC phosphorylates the serine at residue 329 in the cytoplasmic domain of DNAM-1 and increases avidity of DNAM-1-mediated adhesion with cells bearing its ligand (Shibuya et al., 1998) suggested that phosphorylated S^{329} may be involved in the association of DNAM-1 with LFA-1. To test this hypothesis, Jurkat cells were transfected with wild-type *DNAM-1* cDNA (WT) or a site-

directed mutant of *DNAM-1* at S^{329} (S-F^{329}), both tagged with the Flag peptide epitope at the N-terminus. The transfectants were examined for the association of LFA-1 with endogenous and transfected DNAM-1 molecules. Fig. 4B shows that anti-LFA-1 co-immunoprecipitated both endogenous and transfected wild-type DNAM-1. By contrast, the mutant S-F^{329} DNAM-1 glycoprotein was not co-immunoprecipitated with LFA-1. To confirm the phosphorylation of S^{329} of DNAM-1, the Jurkat transfectants were metabolically labeled with [32P]orthophosphate and DNAM-1 proteins were immunoprecipitated with anti-Flag mAb. As shown in Figure 4C, the DNAM-1 proteins in Jurkat cells transfected with WT *DNAM-1*, but not with S-F^{329} mutant *DNAM-1*, were phosphorylated, consistent with the conclusion that LFA-1 associates with wild-type DNAM-1, but not with S-F^{329} mutant DNAM-1. These results indicate that phosphorylated S^{329} plays a critical role in the association of LFA-1 with DNAM-1.

Cross-linking of LFA-1 induces tyrosine phosphorylation of DNAM-1 and fyn in activated T cells

Because the cytoplasmic domain of DNAM-1 contains tyrosines which are phosphorylated by stimulation with pervanadate in T cells and NK cells (data not shown), we examined whether directly cross-linking LFA-1 induces tyrosine phosphorylation of DNAM-1. Fig 5A demonstrates that ligation of LFA-1 with anti-CD18 mAb significantly induced tyrosine phosphorylation of DNAM-1 in anti-CD3 activated T cells, whereas it did not induce the phosphorylation of DNAM-1 in resting, non-stimulated T cells, where LFA-1 is not associated with DNAM-1 (Fig. 3A). It should be noted that low levels of tyrosine phosphorylated DNAM-1 were also detected in T cells activated with anti-CD3 mAb alone (Fig. 5A), possibly because the activated T cells expressed not only LFA-1, but also its ligand ICAM-1. These results suggest that cross-

linking of LFA-1 with antibody or possibly with its ligand results in tyrosine phosphorylation of DNAM-1 in activated T cells.

These results led us to search for the protein tyrosine kinases (PTK) involved in the phosphorylation of DNAM-1. The amino acid sequence REDIY^{322}VNY in the cytoplasmic domain of DNAM-1 is a possible phosphorylation site for a src-family PTK (Shibuya et al., 1996). Therefore, we looked for a src family PTK expressed in T cells that was phosphorylated in response to cross-linking of LFA-1. No PTK tested was phosphorylated by anti-LFA-1 ligation using resting, non-stimulated T cells (Fig 5A and not shown). Stimulation of resting T cells with anti-CD3 mAb alone induced tyrosine phosphorylation of lck and fyn, consistent with the previous reports that these PTK are regulated by the CD3/TcR complex (Iwashima et al., 1994; Samelson et al., 1990). However, co-ligation of both LFA-1 and CD3 induced or augmented tyrosine phosphorylation of fyn, but not of lck, implicating the fyn protein tyrosine kinase in the activation process.

DNAM-1 binds fyn

To address the relationship of fyn with LFA-1 and DNAM-1, resting T cells were stimulated with anti-CD3 and/or pervanadate, were lysed and immunoprecipitated with either anti-CD18 or anti-DNAM-1. As shown in Fig 5B, fyn was co-immunoprecipitated with DNAM-1, but not with CD18, in T cells treated with pervanadate, indicating that fyn is primarily recruited by DNAM-1, but not LFA-1. Fyn was also co-immunoprecipitated with DNAM-1 from T cells activated by anti-CD3 (Fig 5B), consistent with the results that tyrosine phosphorylation of fyn was induced by anti-CD3 stimulation alone (Fig 5A). Because fyn was co-immunoprecipitated with LFA-1 only under conditions that induced association of LFA-1 and DNAM-1, it is likely that this kinase is interacting with DNAM-1 rather than LFA-1, although we can not exclude intermediate adapter molecules in the process.

Fyn phosphorylates Y^{322} of DNAM-1

To determine the site of phosphorylated tyrosine in the DNAM-1 cytoplasmic domain, Jurkat cells were transfected with wild type (WT) or a site-directed mutant (Y-F^{322}) *DNAM-1* , both tagged with Flag at N-terminus. These transfectants were stimulated with pervanadate and the Flag-tagged wild-type and Y-F^{322} DNAM-1 proteins were immunoprecipitated with anti-Flag mAb and then immunoblotted with anti-phosphotyrosine or anti-fyn. Fig 6 shows that phosphorylated tyrosines were detected in wild-type Flag-tagged DNAM-1, but not in Y-F^{322} DNAM-1, indicating that Y^{322} of DNAM-1 is the site phosphorylated in response to pervanadate stimulation. Of note, fyn was co-immunoprecipitated with both wild-type and Y-F^{322} mutant DNAM-1 protein, suggesting that fyn may phosphorylate the Y^{322} of DNAM-1.

To examine whether fyn is responsible for phosphorylation of the tyrosine at residue 322 of DNAM-1, COS-7 cells were co-transfected with wild-type or T-F322 mutant *DNAM-1* and either with wild-type, kinase dead, or constitutively active *fyn* and DNAM-1 proteins were immunoblotted with anti-phosphotyrosine. As demonstrated in Fig 7, the wild-type DNAM-1 was tyrosine phosphorylated in the transfectant with wild-type and constitutively active, but not kinase dead, *fyn*. By contrast, the tyrosine phopshorylation of Y-F322 mutant DNAM-1 was not detected in the transfectant with any type of *fyn*. These results indicate that fyn is likely a responsible kinase for phosphorylation of Y^{322} of DNAM-1.

Discussion

In this study, we demonstrate that initiation of cytotoxicity function by cross-linking the DNAM-1 receptor is dependent upon a functional interaction with LFA-1. Genetic restoration of LFA-1 expression in NK cells established from a LAD patient rendered the DNAM-1 molecule functional, thus providing formal evidence for this association. This observation prompted further studies to understand the molecular basis for this phenomenon. We have shown that LFA-1 physically associates with DNAM-1 in normal peripheral NK cells. Moreover, stimulation of resting T cells with either anti-CD3 or PMA induces the association of LFA-1 and DNAM-1 and this requires phosphorylation of S^{329} in DNAM-1. Presently, it is uncertain whether this reflects a direct interaction or an indirect association via adaptor molecules or the cytoskeleton. Nonetheless, once LFA-1 and DNAM-1 associate, cross-linking LFA-1 results in the tyrosine phosphorylation of DNAM-1. DNAM-1 phosphorylation on tyrosine was not induced by cross-linking DNAM-1 with mAb but requires the ligand binding of LFA-1 (Fig 1C and our unpublished data). We have demonstrated that cross-linking LFA-1 also augmented tyrosine phosphorylation of fyn and this kinase is likely responsible for DNAM-1 phosphorylation at residue 322. Taken together, these results suggest that initiation of cytotoxicity function by cross-linking the DNAM-1 receptor requires tyrosine phosphorylation of DNAM-1 induced by fyn which is activated by LFA-1. Further studies using a genetic reconstitution model will be required to determine whether fyn is involved in the function of DNAM-1 receptor.

The signaling pathways involved in LFA-1 function are complex and incompletely understood. Prior studies have demonstrated that the LFA-1-mediated cell adhesion is enhanced in T cells after stimulation of the TCR or upon activation of PKC by phorbol esters (van Kooyk et al., 1989; Dustin and Springer, 1989). We have previously reported that PKC activation by PMA also increases ligand binding activity of DNAM-1 (Shibuya et al., 1998). In this

study, we have shown that, under these conditions that activate T cells, these adhesion molecules form a physical complex. At present, it is uncertain how are adhesion and intracellular signaling mediated by the complex of LFA-1 and DNAM-1 regulated following activation of T cells. To address this issue, it is essentially required to identify the DNAM-1 ligand and determine the structural requirement involved in DNAM-1 binding.

There are evidences that β1 (α1β1 and α5β1) and β3 (αIIbβ3 and αVβ3) integrins associate with a membrane adaptor molecule caveolin-1 (Wary et al., 1998) and CD47 (Lindberg et al., 1993), respectively. Our studies indicate the physical and functional association of DNAM-1with αLβ2 integrin LFA-1. Many signal transducing molecules are also involed in LFA-1 function. For example, cross-linking of LFA-1 by antibodies or potentially ligand results in the activation of protein tyrosine kinases in T cells or NK cells (Sugie et al., 1995, Kanner, 1996, Rodriguez-Fernandezthe et al., 1999), the stimulation of PLC-γ1 (Kanner et al., 1993) and there are evidence for co-immunoprecipitation of LFA-1 with ZAP70 (Soede et al., 1998) and with cytohesin (Kolanus et al., 1996) in activated T cells. In addition, co-clustering has been demonstrated for the LFA-1-FcγRIIIb pair on neutrophils (Zhou et al., 1993). Furthermore, LFA-1-dependent activation results in translocation of PKC to the cytoskeleton in T cells (Volkov et al., 1998). Further studies will be required to determine the relationship of these signal transducing molecules with DNAM-1 and fyn to elucidate the role of these molecules in LFA-1 function.

Experimental Procedures

Cells and Reagents. Resting T cells were isolated from the peripheral blood of healthy volunteers, as described previously (Azuma et al., 1992). Resting CD3-CD56+ NK cells (CD3-CD56+) were purified by cell sorting using flow cytometry from the peripheral blood of healthy volunteers. NK clones were established from the peripheral blood of healthy donors or a patient with leukocyte adhesion molecule deficiency using the culture conditions described previously (Yssel et al., 1984) and 100 IU/ml recombinant IL-2 as a growth factor. Jurkat is a human T cell leukemia cell line. NKL is a human NK leukemia cell line generously provided by Dr. Mike Robertson (U. Indianapolis) (Robertson et al., 1996). P815 is a murine mastocytoma cell line. PMA was purchased from Sigma (St. Louis, Missouri) and dissolved in DMSO, which did not exceed 0.1% in the assay medium.

Abs and Flow Cytometry. Control IgG, anti-CD3 (Leu 4 mAb), anti-CD16 (Leu 11a mAb), anti-CD11a (L7 mAb), anti-CD11b (Leu 15 mAb), anti-CD11c (Leu M5 mAb), anti-CD56 (Leu 19 mAb), and anti-CD18 (L130 mAb) were generously provided by Becton Dickinson Immunocytometry Systems (San Jose, CA). Anti-DNAM-1 mAb (DX11) were generated as described (Shibuya et al., 1996). Anti-phosphotyrosine mAb (4G10) was purchased from Upstate Biotechnology Inc., Lake Placid, NY. Anti-Flag mAb was purchased from Sigma. Anti-protein tyrosine kinases lck and fyn are polyclonal rabbit Abs that were gifts from Dr. Joe Bolen (DNAX). $F(ab')_2$ fragments of anti-CD18 were prepared as described (Shibuya et al., 1996). Purity of the $F(ab')_2$ fragments were determined by SDS-PAGE. Methods of immunofluorescent staining and flow cytometry have been described previously (Lanier and Recktenwald, 1991).

Cytotoxicity assay. Tumor cell lines (~ 1.5 x 10^6 cells) were labeled with 100 uCi ^{51}Cr for 2 h, washed, and used as targets in a 4-h radioisotope release cytotoxicity assay, as described (Lanier et al., 1983). Data are expressed as the means of triplicate cultures. Spontaneous radioisotope release was typically <10% of total ^{51}Cr release. Percent cytolysis was calculated as:

$$\frac{\text{cpm specific } ^{51}\text{Cr release} - \text{cpm spontaneous release}}{\text{cpm total } ^{51}\text{Cr release} - \text{cpm spontaneous release}} \times 100$$

Genetic reconstitution of LAD NK cell clones. A cDNA containing the open reading frame of CD18 was generated by PCR using a wild-type CD18 cDNA plasmid as the template. The PCR products were subcloned into a retroviral vector pMX-neo (Onishi et al. , 1996) (kindly provided by Toshio Kitamura, DNAX) with cloning sites of Bam H1(5') and Not 1 (3'). Phoenix-amphotropic packaging cells (Kinsella and Nolan, 1996) (generously provided by Gary Nolan, Stanford) were transfected with the CD18 cDNA in the retroviral vector using lipofectamine (GibcoBRL), as described previously (Kitamura et al., 1995, Onishi et al., 1996). LAD NK clones were infected with the CD18 retrovirus stock. Two days after infection, the cells were analyzed and CD18 expressing cells were sorted by flow cytometry and expanded in the culture conditions described previously (Yssel et al., 1984). Expression of CD18, CD11a, CD11b, and CD11c were analyzed by flow cytometry.

Establishment of Jurkat transfectants with wild type and mutant DNAM-1. Wild type DNAM-1 cDNA was subcloned into the pFLAG-CMV-1 vector (Scientific Imaging Systems, Rochester, NY) with cloning sites of Sal 1 (5') and Not 1 (3'). To generate site-specific DNAM-1 mutants at residues Y^{322} or S^{329}, antisense PCR primers, which contained a codon for F (TTT) instead of Y^{322} (TAT) or S^{329} (TCT), were designed. The PCR products were

subcloned into the pFLAG-CMV-1 vector with the same cloning sites as the wild type cDNA. All mutant cDNAs were verified by sequencing. Jurkat cells were transfected with the wild type or site-specific mutant DNAM-1 using DMRIE-C (GibcoBRL, Gaithersburg, MD). Expression of wild type and mutant DNAM-1 were analyzed by flow cytometry using anti-Flag mAb (Sigma).

Establishment of COS-7 co-transfectants with DNAM-1 and fyn. COS-7 cells were co-transfected with the wild type or site-specific mutant DNAM-1, described above, together with either wild type, kinase dead (having the site-directed mutation of M instead of K at residue 296) or constitrutively active (having the site-directed mutation of F instead of Y at residue 528) (Takeuchi et al., 1993) fyn (kindly provided by Tadashi Yamamoto, University of Tokyo) using DMRIE-C. Expression of DNAM-1 and fyn were analyzed by immunoblotting.

Biochemistry. To examine the association of molecules with LFA-1 or DNAM-1, cells were stimulated or not with plastic-precoated mAbs for 2 min, and/or 100 mM sodium pervanadate for 10 min at 37ºC (O'Shea et al., 1992). Cells were lysed in 1% digitonin (Sigma) buffer ((0.12% triton-100 (Katayama Pure Chemical, Osaka, Japan), 150 mM NaCl, 20 mM triethanolamine (Katayama Pure Chemical), protease inhibitors (1 mM PMSF and 20 Kallikrein inhibitor U/ml aprotinin) and phosphatase inhibitors (1 mM EGTA, 10 mM NaF, 1 mM Na_4PO_7, 0.1 mM β-glycerophosphatase, 1 mM Na_3VO_4)). The lysates were immunoprecipitated with anti-CD18, anti-DNAM-1 (DX11 mAb), anti-Flag or control Ig and proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Immunoprecipitates were transferred (100V, 1 hr in 25 mM Tris, 195 mM glycine, 20% methanol) to PVDF membranes (Immobilon-P, Millipore). Membranes were incubated overnight in Tris buffered saline (TBST) containing 0.5% Tween 20, $MgCl_2$ and 3% BSA at 4ºC, and then incubated with primary antibodies (1 ug/ml in TBST with 3% BSA) for 2 hr at room

temperature. Proteins were detected by using HRP-conjugated goat anti-mouse or anti-rabbit Ig (Amersham, Arlington Heights, IL) and developed with SuperSignal CL-HRP Substrate (Pierce, Rockford, IL). Chemiluminescence was detected by autoradiograph.

To examine tyrosine phosphorylation of DNAM-1, cells were stimulated or not with plastic-precoated mAbs for 2 min. and/or 100 mM sodium pervanadate for 10 min at 37ºC. Cells were lysed in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 8.0) containing 1% NP-40, and the protease and phosphatase inhibitors described above. Cell lysates were immunoprecipitated with control Ig, anti DNAM-1 mAb (DX11), anti-Flag or anti-PTKs lck or fyn and proteins were separated by SDS-PAGE. Immunoprecipitates were immunoblotted, as described above, and then incubated with anti-phosphotyrosine 4G10 (1 ug/ml in TBST with 3% BSA) for 2 hr at room temperature. Proteins were detected by using HRP-conjugated goat anti-mouse Ig.

For the analysis of tyrosine phosphorylation of DNAM-1 induced by mAbs and P815 cells, P815 cells were pre-coated with control Ig or anti-DNAM-1 (DX11 mAb) and then incubated with NK clones derived from a LAD patient, as described previously (Shibuya et al., 1996).

Acknowledgment

We thank Jacques Chiller for encouraging us to do this research. We also thank Junko Mizuuchi and Michie Ito for their secretarial assistance, Midori Isobe for technical assistance, Yohei Morita and Reiko Shimamura for expert assistance with flow cytometry, and Masafumi Onodera and Atsushi Iwama for discussion. This research was supported in part by the grants provided by the Ministry of Education, Science and Culture of Japan, the Osaka Cancer Institute, the Ichiro Kanehara Foundation, the Mochida Memorial Foundation, the Ryoichi Naito Foundation, the Asahi Glass Foundation and Schering-Plough Corporation (Osaka, Japan) (to A.S.) and the Uehara Memorial Foundation (to

K.S.). DNAX Research Institute is supported by Schering-Plough Corporation (NJ).

References

Anderson, D. C., and Springer, T. A. (1987). Leukocyte adhesion deficiency: An inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. Ann. Rev. Med. *38*, 175-194.

Appleby, M. W., Gross, J. A., Cooke, M. P., Levin, S. D., Qian, X., and Perimutter, R. M. (1992). Defective T cell receptor signaling in mice lacking the thymic isoform of p59^{fyn}. Cell *70*, 751-763.

Arnaout, M. A. (1990). Leukocyte adhesion molecules deficiency: its structual basis, pathophysioplogy and implications for modulating the inflammatory response. Immunol. Rev. *114*, 145-181.

Arroyo, A., Campanero, M., Sanchez-Mateos, P., Zapata, J., Ursa, M., del Pozo, M., and Sanchez-Madrid, F. (1994). Induction of tyrosine phosphorylation during ICAM-3 and LFA-1-mediated intercellular adhesion, and its regulation by the CD45 tyrosine phosphatase. J. Cell Biol. *126*, 1277-1286.

Azuma, M., Cayabyab, M., Buck, D., Phillips, J. H., and Lanier, L. L. (1992). CD28 interaction with B7 co-stimulates primary allogeneic proliferative responses and cytotoxicity mediated by small, resting T lymphocytes. J. Exp. Med. *175*, 353-360.

Bauer, T. R., Barbara R, J., Schwartz, B. R., Liles, W. C., Ochs, H. D., and Hickstein, D. D. (1998). Retroviral-mediated gene transfer of the leukocyte integrin CD18 into peripheral blood CD34+ cells derived from a patient with leukocyte adhesion molecule deficiency type 1. Blood *91*, 1520-1526.

Cantrell, D. (1996). T cell antigen receptor signal transduction pathways. Ann. Rev. Immunol. *14*, 259-274.

Cayabyab, M., Phillips, J. H., and Lanier, L. L. (1994). CD40 preferentially costimulates activation of CD4+ T lymphocytes. J. Immunol. *152*, 1523-1531.

Dransfield, I., Cabanas, C., Craig, A., and Hogg, N. (1992). Divalent cation regulation of the function of the leukocyte integrin LFA-1. J. Cell Biol. *116*, 219-226.

Dustin, M. L., Carpen, O., and Springer, T. A. (1992). Regulation of locomotion and cell-cell contact area by the LFA-1 and ICAM-1 adhesion receptors. J. Immunol. *148*, 2654-2663.

Dustin, M. L., and Springer, T. A. (1991). Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. Ann. Rev. Immunol. *9*, 27-66.

Dustin, M. L., and Springer, T. A. (1989). T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature *341*, 619-624.

Fawcett, J., Holness, C. L., Needham, L. A., Turley, H., Gatter, K. C., Mason, D. Y., and Simmons, D. L. (1992). Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes. Nature *360*, 481-484.

Hibbs, M. L., Wardlaw, A. J., Stacker, S. A., Anderson, D. C., Lee, A., Roberts, T. M., and Springer, T. A. (1990). Transfection of cells from patients with leukocyte adhesion deficiency with an integrin b subunit (CD18) restores lymphocyte function-associated antigen-1 expression and function. J. Clin. Invest. *85*, 674-681.

Hogg, N., and Landis, R. C. (1993). Adhesion molecules in cell interactions. Curr Opin in Immunol *5*, 383-390.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11-25.

Isobe, M., Yagita, H., Okumura, K., and Ihara, A. (1992). Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. Science *255*, 1125-1127.

Iwashima, M., Irving, B. A., van Oers, N. S. C., Chang, A. C., and Weis, A. (1994). Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. Science *263*, 1136-1139.

Kanner, S.B., Grosmaire, L.S., Ledbetter, J.A., Damle, N.K. (1993) Beta 2-integrin LFA-1 signaling through phospholipase C-gamma 1 activation. Proc. Natl. Acad. Sci. USA *90*:7099-103

Kanner, S.B. (1996) Focal adhesion kinase-related fakB is regulated by the integrin LFA-1 and interacts with the SH3 domain of phospholipase C gamma 1. Cell. Immunol. *171*:164-9.

Kinsella, T.M., and Nolan, G.P. (1996) Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. Hum Gene Ther *7*:1405-13

Kishimoto, T., Hollander, N., Roberts, T., Anderson, D., and Springer, T. (1987). Heterogeneous mutations in the beta subunit common to the LFA-1, Mac-1, and p150,95 glycoproteins cause leukocyte adhesion deficiency. Cell *50*, 193-202.

Kitamura, T., Onishi, M., Kinoshita, S., Shibuya, A., Miyajima, A., and Nolan, G. P. (1995). Efficient screening of retroviral cDNA expression libraries. Proc Natl Acad Sci Usa *92*, 9146-9150.

Kolanus, W., Nagel, W., Schiller, B., Zeitlmann, L., Godar, S., Stockinger, H., and Seed, B. (1996). $\alpha L\beta 2$ integrin/LFA-1 binding to ICAM-1 induced by cytohesin, a cytoplasmic regulatory molecule. Cell *86*, 233-242.

Koopman, G., Keehnen, R., Lindhout, E., Newman, W., Shimizu Y, v., Seventer, G., de Groot, C., and Pals, S. (1994). Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106)

pathways prevents apoptosis of germinal center B cells. J Immunol *152*, 3760-3767.

Krensky, A., Mentzer, S., Clayberger, C., Anderson, D., Schmalstieg, F., Burakoff, S., and Springer, T. (1985). Heritable lymphocyte function-associated antigen-1 deficiency: abnormalities of cytotoxicity and proliferation associated with abnormal expression of LFA-1. J. Immunol. *135*, 3102-3108.

Lanier, L. L., Corliss, B., and Phillips, J. H. (1997). Arousal and inhibition of human NK cells. Immunol. Rev. *155*, 145-154.

Lanier, L. L., Le, A. M., Phillips, J. H., Warner, N. L., and Babcock, G. F. (1983). Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. J. Immunol. *131*, 1789-1796.

Lanier, L. L., and Recktenwald, D. J. (1991). Multicolor immunofluorescence and flow cytometry. Methods: A Companion to Methods in Enzymology *2*, 192-199.

Lindberg, F.P., Gresham, H.D., Schwarz, E., Brown, E.J. (1993) Molecular cloning of integrin-associated protein: an immunoglobulin family member with multiple membrane-spanning domains implicated in alpha v beta 3-dependent ligand binding. J. Cell. Biol.*123*,485-96

Moy, V., and Brian, A. (1992). Signaling by lymphocyte function-associated antigen 1 (LFA-1) in B cells: enhanced antigen presentation after stimulation through LFA-1. J Exp Med *175*, 1-7.

O'Shea, J. J., McVicar, D. W., Bailey, T. L., Burns, C., and Smyth, M. J. (1992). Activation of human peripheral blood T lymphocytes by pharmacological induction of protein-tyrosine phosphorylation. Proc. Natl. Acad. Sci. USA *89*, 10306-10310.

Onishi, M., Kinoshita, S., Morikawa, Y., Shibuya, A., Phillips, J. H., Lanier, L. L., Gorman, D. M., Nolan, G. P., Miyajima, A., and Kitamura, T. (1996). Application of retrovirus-mediated expression cloning. Exp Hematol *24*, 324-329.

Robertson, M. J., Cochran, K. J., Cameron, C., Le, J.-M., Tantravahi, R., and Ritz, J. (1996). Characterization of a cell line, NKL, derived from an aggressive human natural killer cell leukemia. Exp. Hematol. *24*, 406-415.

Rodriguez-Fernandez, J.L., Gomez, M., Luque, A., Hogg, N., Sanchez-Madrid, F., Cabanas,C. (1999) The interaction of activated integrin lymphocyte function-associated antigen 1 with ligand intercellular adhesion molecule 1 induces activation and redistribution of focal adhesion kinase and proline-rich tyrosine kinase 2 in T lymphocytes. Mol. Biol. Cell.*10*:1891-907

Samelson, L. E., Phillips, A. F., Luong, E. T., and Klausner, R. D. (1990). Association of the fyn protein-tyrosine kinase with the T-cell antigen receptor. Proc. Natl. Acad. Sci. USA *87*, 4358-4362.

Scharffetter-Kochanek, K., Lu, H., Normann, K., van Nood, N., Munoz, F., Grabbe, S., McArthur, M., Lorenzo, I., Kaplan, S., Ley, K., Smith, C. W., Montogomery, C. A., Rich, S., and Beaudet, A. L. (1998). Spontaneous skin ulcerlation and defective T cell function in CD18 null mice. J. Exp. Med. *188*, 119-131.

Schmidt, R.E., Hercend, T., Fox, D.A., Bensussan, A., Bartley, G., Daley, J.F., Schlossman, S.F., Reinherz, E.L., Ritz, J. (1985) The role of interleukin 2 and T11 E rosette antigen in activation and proliferation of human NK clones. J. Immunol. *135*:672-8

Schmits, R., Kundig, T. M., Baker, D. M., Shumarker, G., Simard, J. J. L., Duncan, G., wakeham, A., Shahinian, A., van der Heiden, A., Bachmann, M. F., Ohashi, P. S., Mak, T. W., and Hickstein, D. D. (1996). LFA-1-deficient mice show normal CTL responses to virus but fail to reject immunologic tumor. J. Exp. Med. *183*, 1415-1426.

Shibuya, A., Campbel, D., Hannum, C., Yssel, H., Franz-Bacon, K., McClanahan, T., Kitamura, T., Nicoll, J., Sutherland, G. R., Lanier, L. L., and Phillips, J. H. (1996). DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. Immunity *4*, 573-581.

Shibuya, A., Lanier, L. L., and Phillips, J. H. (1998). Protein kinase C is involved in the regulation of both signaling and adhesion mediated by DNAM-1 receptor. J. Immunol. *161*, 1671-1676.

Shuster, D. E., Kehrli, J., M.E.,, Ackermann, M. R., and Gibert, R. O. (1992). Identification and prevalence of a genetic defect that causes leukocyte adhesion deficiency in Holstein cattle. Proc. Natl. Acad. Sci. USA. *89*, 9225-9229.

Siliciano, R.F., Pratt, J.C., Schmidt, R.E., Ritz, J., Reinherz, E.L. (1985) Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. Nature *317*:428-30

Soede,R.D., Wijnands, Y.M., Van Kouteren-Cobzaru, I., Roos, E. (1998) ZAP-70 tyrosine kinase is required for LFA-1-dependent T cell migration. J. Cell. Biol. *142*:1371-1379

Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991). Targeted disruption of the c-src protooncogene leads to osteoporosis in mice. Cell *64*, 693-702.

Springer, T. A. (1990). Adhesion receptors of the immune system. Nature *346*, 425-434.

Springer, T. A., Dustin, M. L., Kishimoto, T. K., and Marlin, S. D. (1987). The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: Cell adhesion receptors of the immune system. Ann. Rev. Immunol. *5*, 223-252.

Staunton, D. E., Dustin, M. L., and Springer, T. A. (1989). Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature *339*, 61-64.

Staunton, D. E., Marlin, S. D., Stratowa, C., Dustin, M. L., and Springer, T. A. (1988). Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. Cell *52*, 925-933.

Stein, P. L., Lee, H.-M., Rich, S., and Soriano, P. (1992). pp59^{fyn} mutant mice display differential signaling in thymocytes and peripheral T cells. Cell *70*, 741-750.

Sugie, K., Minami, Y., Kawakami, T., Uchida, A. (1995) Stimulation of NK-like YT cells via leukocyte function-associated antigen (LFA)-1. Possible involvement of LFA-1-associated tyrosine kinase in signal transduction after recognition of NK target cells. J. Immunol.*154*:1691-8

Takeuchi, M., Kuramochi, S., Fusaki, N., Nada, S., Kawamura-Tsuzuku, J., Matsuda, S., Semba, K., Toyoshima, K., Okada, M., Yamamoto, T. (1993) Functional and physical interaction of protein-tyrosine kinases Fyn and Csk in the T-cell signaling system. J. Biol. Chem *268*:27413-27419

Timonen, T., Patarroyo, M., Gahmberg, C.G. (1988) CD11a-c/CD18 and GP84 (LB-2) adhesion molecules on human large granular lymphocytes and their participation in natural killing. J. Immunol. *141*:1041-6

van Kooyk, Y., van de Wiel-van Kemenade, P., Weder, P., Kuijpers, T. W., and Figdor, C. G. (1989). Enhancement of LFA-1 mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes. Nature *342*, 811-813.

van Kooyk, Y., Weder, P., Hogervorst, F., Verhoeven, A. J., van Seventer, G., te Velde, A. A., Borst, J., Keizer, G. D., and Figdor, C. G. (1991). Activation of LFA-1 through a Ca^{2+}-dependent epitope stimulates lymphocyte adhesion. J. Cell Biol. *112*, 345-354.

Volkov Y, Long A, Kelleher D (1998) Inside the crawling T cell: leukocyte function-associated antigen-1 cross-linking is associated with microtubule-directed translocation of protein kinase C isoenzymes beta(I) and delta. J. Immunol. *161*,6487-95

Wary, K.K., Mariotti, A., Zurzolo, C., Giancotti, G. (1998) A requirement for caveolin-1 and associated kinase fyn in integrin signaling and anchorage-dependent cell growth. Cell *94*, 625-634

Yssel, H., De Vries, J. E., Koken, M., van Blitterswijk, W., and Spits, H. (1984). Serum-free medium for the generation and the propagation of functional human cytotoxic and helper T cell clones. J. Immunol. Methods *72*, 219-227.

Zhou, M., Todd, R.F. 3d., van de Winkel, J.G., Petty, H.R. (1993) Cocapping of the leukoadhesin molecules complement receptor type 3 and lymphocyte function-associated antigen-1 with Fc gamma receptor III on human neutrophils. Possible role of lectin-like interactions. J Immunol *150*, 3030-41

Figure Legends

Fig 1. NK cell clones from LAD patients are deficient in anti-DNAM-1 induced cell-mediated cytotoxicity

(A) CD3-CD56+ NK clones were established from a patient with Leukocyte Adhesion Deficiency (LAD). The LAD NK clones lacked the expression of CD11a, CD11b, CD11c, and CD18 (i.e. LFA-1, Mac-1, and p150/95) (left). The CD18 cDNA was introduced in the LAD NK clone using an amphotropic retrovirus and NK cells stably expressing cell surface LFA-1 were sorted by flow cytometry and expanded. Genetic transfer of *CD18* into the LAD NK clone restored the expression of CD11a and CD18 (i.e. LFA-1) but these cells did not express CD11b or CD11c (i.e. MAC-1 and p150/95) (right).

(B) NK clones from a healthy donor killed the Fc receptor-bearing P815 target in the presence of anti-DNAM-1 mAb (left panel). LAD NK cell clones killed the P815 target in the presence of anti-CD16 mAb, but not anti-DNAM-1 mAb (center). *CD18* gene transfer into the LAD NK clone restored anti-DNAM-1 mAb-induced cytolysis. Anti-DNAM-1 induced killing was inhibited in the presence of $F(ab')_2$ fragments of anti-CD18 mAb (left and right).

(C) The LAD NK clone lacking the expression of LFA-1 (left panel) or the LAD NK clone expressing LFA-1 after *CD18* gene transfer (right) were incubated for 2 min with P815 cells pre-coated with each mAb indicated. Cells were then lysed in 1% NP-40 buffer and immunoprecipitated with anti-DNAM-1. The immunoprecipitates were analyzed by immunoblot using anti-phosphotyrosine (4G10) (upper) or anti-DNAM-1 mAb (lower). Tyrosine phosphorylation of DNAM-1 was not induced in the LAD NK clone (left), but was observed in the LAD clone expressing LFA-1 (right). The tyrosine phosphorylation of DNAM-1 in the CD18 reconstituted LAD NK cell clone was abolished in the presence of $F(ab')_2$ fragments of anti-CD18 mAb (right).

Fig 2. Association of LFA-1 with DNAM-1 in peripheral NK cells

Resting peripheral blood CD3-CD56+ NK cells were lysed in 1% digitonin buffer and immunoprecipitated (IP) with control Ig, anti-CD18, CD11a, CD11b, CD11c or anti-DNAM-1. The immunoprecipitates were analyzed by immunoblotting (IB) with anti-DNAM-1 mAb. DNAM-1 was co-immunoprecipitated with CD11a and CD18, but not with CD11b and CD11c.

Fig 3. Anti-CD3 or PMA induced association of LFA-1 with DNAM-1 in peripheral T cells

(A) Resting peripheral blood T cells were stimulated with plastic-coated cIg or anti-CD3 mAb for 2 min in the presence or absence of the PKC inhibitor GF109203X (1uM), or PMA (50 ng/ml) at 37^{o}C for 2 hours. The cells were lysed in 1% digitonin buffer and immunoprecipitated with anti-CD18 mAb or control Ig. The immunoprecipitates were analyzed by immunoblotting with anti-DNAM-1 mAb. CD18 co-immunoprecipitates DNAM-1 in T cells activated by anti-CD3 or PMA.

(B) Resting peripheral T cells were metabolically labeled with [^{32}P]orthophosphate, stimulated with cIg, anti-CD3 mAb or PMA, lysed, and immunoprecipitated with anti-DNAM-1 mAb. Treatment of resting T cells with either anti-CD3 mAb or PMA resulted in phosphorylation of DNAM-1.

(C) Resting peripheral blood T cells were stimulated with plastic-coated control Ig or anti-CD3 mAb for 2 min. The cells were stimulated or not with pervanadate for 10 min at 37^{o}C and lysed in the 1% digitonin buffer. The lysates were immunoprecipitated with anti-CD18 mAb or control Ig and analyzed by immunoblotting with anti-DNAM-1 mAb. DNAM-1 was co-immunoprecipitated with LFA-1 in T cells activated by anti-CD3, but not in resting T cells, regardless of the stimulation with pervanadate.

Fig. 4 Role of Ser^{329} of DNAM-1 in the association of DNAM-1 with LFA-1

(A) Jurkat cells and NKL cells were lysed in the 1% digitonin lysis buffer and immunoprecipitated with anti-DNAM-1 mAb, anti-CD18 mAb or cIg. The immunoprecipitates were analyzed by immunoblotting with anti-DNAM-1 mAb. DNAM-1 was co-immunoprecipitated with CD18 in Jurkat and NKL cells.

(B) Jurkat cells were transfected with wild type or site-directed mutant (S-F^{329}) *DNAM-1*, both tagged with the Flag epitope. The transfectants were lysed in the 1% digitonin buffer and immunoprecipitated with anti-CD18 mAb or control Ig. The immunoprecipitates were analyzed by immunoblotting with anti-DNAM-1 mAb or anti-Flag mAb. CD18 was co-immunoprecipitated with wild type DNAM-1, but not with S-F^{329} DNAM-1.

(C) Jurkat transfectants with wild type or site-directed mutant (S-F^{329}) *DNAM-1* were metabolically labeled with [32P]orthophosphate and DNAM-1 proteins were immunoprecipitated with anti-Flag mAb. The DNAM-1 proteins in Jurkat cells transfected with WT *DNAM-1*, but not with S-F^{329} mutant *DNAM-1*, were phosphorylated.

Fig 5. Relationship between LFA-1, DNAM-1, and the protein tyrosine kinase fyn

(A) Resting peripheral blood T cells were stimulated with plastic-coated cIg, anti-CD3 mAb and/or anti-CD18 mAb for 2 min. The cells were then lysed in the 1% NP-40 buffer and immunoprecipitated with cIg, anti-protein tyrosine kinases lck, -fyn, or anti-DNAM-1 mAb. The immunoprecipitates were immunoblotted with anti-phosphotyrosine mAb 4G10. Tyrosine phosphorylation of fyn and DNAM-1 was augmented by the stimulation with anti-CD18 in the combination with anti-CD3, but not by cross-linking with anti-CD18 alone.

(B) Resting peripheral blood T cells were stimulated with plastic-coated cIg or anti-CD3 mAb for 2 min. Then the cells were stimulated or not with

pervanadate for 10 min at 37ºC and lysed in 1% digitonin buffer. The lysates were immunoprecipitated with cIg, anti-CD18 or anti-DNAM-1 and analyzed by immunoblotting with anti-fyn. Fyn was co-immunoprecipitated with DNAM-1, but not with CD18, in T cells stimulated with pervanadate alone. Co-immunoprecipitation of fyn with CD18 was always detected in T cells stimulated with anti-CD3 mAb.

Fig 6. Y^{322} is required for pervanadate-induced phosphorylation of DNAM-1

Jurkat cells were transfected with wild type or site-directed mutant $Y\text{-}F^{322}$ *DNAM-1*, both tagged with the Flag epitope. Two days after transfection, Jurkat cells were stimulated with pervanadate for 10 min at 37ºC and lysed in 1% NP-40 buffer (left) or 1% digitonin buffer (right). The lysates were immunoprecipitated with anti-Flag or cIg and analyzed by immunoblotting with anti-phosphotyrosine (4G10) or anti-fyn. Wild type DNAM-1, but not $Y\text{-}F^{322}$ DNAM-1, was tyrosine phosphorylated (left). Fyn was co-immunoprecipitated with both wild type and $Y\text{-}F^{322}$ DNAM-1.

Fig 7. Fyn is responsible for tyrosine phophorylation of DNAM-1

COS 7 cells were co-transfected with wild-type or $T\text{-}F^{322}$ mutant *DNAM-1* and either with wild-type, kinase dead, or constitutively active *fyn*. Two days after transfection, DNAM-1 proteins were immunoblotted with anti-phosphotyrosine. The wild-type DNAM-1 protein was tyrosine phosphorylated in the co-transfectant with wild-type and constitutively active, but not kinase dead, *fyn*. By contrast, the tyrosine phopshorylation of $Y\text{-}F^{322}$ mutant DNAM-1 was not detected in the transfectant with any type of *fyn* (right).